Investigations on the toxicological profile of functionalized fifth-generation poly(propylene imine) dendrimer

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Abstract

Dendrimers have generated tremendous interest in the field of drug delivery. Despite indications of their utility as drug carriers, the inherent cytotoxicity associated with polycationic dendrimers acts as a limiting factor to their clinical applications. Many functionalization strategies have been adopted to mask peripheral amines in order to overcome this limitation. The object of the present investigation was to evaluate the effect of functionalization on the toxicological profile of fifth-generation poly(propylene imine) dendrimer (PPI-5.0G). Four forms of functionalized dendrimers, including protected glycine and phenylalanine, and mannose and lactose functionalized poly(propylene imine) (PPI) dendrimer, were synthesized as prospective drug carriers. These dendrimeric systems were evaluated for haemolytic toxicity, cytotoxicity, immunogenicity and haematological parameters. PPI-5.0G demonstrated a positive charge-based time- and concentration-dependent toxicity profile. Functionalization greatly improved the toxicity profile of the parent dendrimer. Hence it is proposed that these functionalized forms of PPI dendrimer have great potential as biocompatible drug vehicles.

Introduction

Dendrimers are novel three-dimensional polymeric architectures that have generated tremendous interest in the field of drug delivery. Their unique characteristics, such as nanometric size, container properties and large number of peripheral functional groups, make them attractive drug carriers. Dendrimers are finding increasing application in the field of drug and gene/DNA delivery (Zinselmeyer et al 2002; Aulenta et al 2003; Kihara et al 2003; Ambade et al 2005; Khandare et al 2005; Kohle et al 2005). Dendrimers with free amine groups at the periphery are reported to show concentration- and generation number-dependent toxicity, which limits their clinical applications (Roberts et al 1996; Wilbur et al 1998; Malik et al 2000). This toxicity was attributed to the positive charge associated with amine-terminated dendrimers. Many functionalization strategies have been used to mask the terminal amine groups and thereby reduce the inherently associated positive charge. Functionalization of dendrimers has also been found to impart to them many other properties beneficial for drug delivery, including modification of their physicochemical properties to make the system more suitable for the proposed application, enhancing the peripheral congestion to improve container properties and attachment of targeting groups to the periphery (Kojima et al 2000; Liu et al 2000; Sideratou et al 2000; Quintana et al 2002; Bhadra et al 2003; Bhadra et al 2005). Poly(propylene imine) (PPI) dendrimer is an amine-terminated dendrimer that is not exempt from the toxicity derived from the terminal amine groups, and therefore its use is constrained. In an attempt to improve the biocompatibility of PPI dendrimer, we decided to mask the peripheral amine groups. These PPI dendrimers with anticipated biocompatibility would be of use only if the synthesized systems have some potential as drug carriers, hence only functionalization schemes that result in dendrimeric systems with drug carrier potential were examined. We synthesized two types of functionalized PPI dendrimers, adopting two different functionalization strategies (Jansen et al 1994; Ashton et al 1997). The first category included t-BOC-protected amino-acid-coated PPI dendrimers and the second category comprised carbohydrate-coated PPI dendrimers. Meijer's group

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(Jansen et al 1994) first reported synthesis of t-BOC-protected amino-acid-coated PPI dendrimer and proposed that the severely congested periphery of these modified dendrimers would impart container properties on them, which might prove useful in controlled drug delivery. The carbohydrate-coated dendrimers were synthesized by Ashton et al (1997). These dendrimers were proposed for targeting of the drug to lectin-rich organs. Thus these selected functionalized dendrimers were expected not only to improve the biocompatibility of the parent PPI dendrimer but also to have potential as drug vehicles.

An ideal drug carrier should be essentially non-toxic and non-immunogenic. Characterization of any polymeric system that is proposed as a drug carrier is incomplete without toxicity assessment. As dendrimers are known to possess charge-dependent toxicity, we decided to undertake a thorough investigation of the toxicity profile of the synthesized functionalized dendrimers that we intend to explore as drug carriers in future experiments. In the present communication we describe the toxicity profiles of four functionalized PPI dendrimers and compare them to that of the parent dendrimer.

The peripheral amino groups of the dendrimers are responsible for the net positive charge of the molecule. This enables the dendrimers to condense negatively charged DNA into globular nanometric size and subsequent transfection. It has been reported that with an increase in positive charge transfection efficiency increases, reaching an optimum value beyond which it once again starts declining. This may be explained on the basis of the fact that with the increase in positive charge (number of unmasked primary amino groups) the transfection efficiency increases until the charge becomes optimally cytotoxic. Beyond this point, a rapid decrease in cell viability reduces the transfection efficiency. Thus, masking of primary amino groups leads to a drastic fall in cytotoxicity and increases the transfection efficiency several fold.

Materials and Methods

Materials

Cell lines
The adherent cell line COS-7 (SV 40 virus transformed African green monkey kidney cell line, ATCC CRL 1651) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (10%), L-glutamine (2 mM), L-glucose (1 gL^{-1}), streptomycin (100 μgmL^{-1}), penicillin (100 IU mL^{-1}) and amphotericin B (0.25 μgmL^{-1}). The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

A human hepatoma cell line, HepG2 (ATCC No. HB-8065), was cultured in minimum essential medium (MEM) supplemented with Earl's salt (1.5 gL^{-1}), fetal calf serum (10%), L-glutamine (2 mM), sodium pyruvate (1%), streptomycin (100 μgmL^{-1}), penicillin (100 IUmL^{-1}) and amphotericin B (0.25 μgmL^{-1}). The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Ethylene diamine and acrylonitrile were purchased from CDH Pvt Ltd (India). t-BOC-protected glycine and t-BOC-protected phenylalanine were purchased from Spectrochem Pvt Ltd (India). Thiopropionic acid, dimethoxyethane and boron trifluoride dietharate (BF3OEt$_2$) were purchased from CDH India Ltd. Raney nickel catalyst was purchased from Merk Schuchardt (Germany). Cellulose dialysis membrane (MWCO 5/12–14 kDa, 2.4 nm pore size) was purchased from HiMedia Laboratories Pvt Ltd (India). Dextran (m. wt 60 000), DMEM, MEM and 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from HiMedia Laboratories Pvt Ltd (India). Horse radish peroxidase, tetramethyl benzidine (TMB) and goat-anti-rat IgG monospecific antisera were purchased from Bangalore Genei (India). All the chemicals were used after distillation. The protocols for animal experiments were duly approved by the Institutional Animal Ethics Committee of the university (Registration Number 379/01/ab/CPCSEA, India).

Synthesis of dendrimeric systems

Poly(propylene imine) dendrimer
PPI dendrimers were synthesized up to fifth generation by the divergent approach reported by De Brabender-Van Den Berg & Meijer (1993). Briefly, ethylene diamine was used as the initiator core. Acrylonitrile was added to it in a double Michael addition reaction to produce half-generation (–CN terminated) dendrimer. Excess acrylonitrile was removed as water azeotrope by vacuum distillation (rotary flask evaporator, Superfit, India). CN-terminated dendrimer was heterogeneously hydrogenated using Raney nickel catalyst to produce full-generation (–NH$_2$ terminated) dendrimer. The reaction sequence was repeated cyclically to produce PPI dendrimer up to fifth generation (PPI-5.0G). Purification of dendrimers was carried out by passing them through a Sephadex G25 mini column followed by HPLC using an octadecyl silane (C$_{18}$) column and UV-visible detector. The presence of a single peak confirms the absolute purity of the synthesized dendrimers.

Functionalized dendrimers
Amino-acid-coated PPI dendrimer was synthesized by reported procedure (Jansen et al 1994). Briefly, activated N-hydroxy succinimide (NHS) esters of t-BOC-protected amino acids (glycine or phenylalanine) were brought into reaction with PPI-5.0G in dichloromethane and triethyl amine at room temperature. After stirring overnight, the reaction mixture was washed with distilled water to remove unreacted PPI-5.0G and dried over Na$_2$SO$_4$. The amino acid functionalized PPI dendrimers were obtained on evaporation of the solvent under vacuum. Carbohydrate-coated PPI dendrimer was synthesized by the procedure reported by Ashton et al (1997). Briefly, the activated NHS ester of acetylated thioglycoside (lactose or mannose) was brought into reaction with PPI-5.0G to form carbohydrate-coated PPI-5.0 dendrimers through thioglycosidic linkage. Deacetylation was carried out by Zemplen reaction. The products were purified by dialysis against water to remove the unreacted reactants (cellulose acetate dialysis membrane, molecular weight cut off 2 kDa). Thus there were four functionalized dendrimers: t-BOC-protected glycine-coated dendrimer (DBG), t-BOC-protected phenylalanine-coated dendrimer (DBPA), mannose-coated dendrimer

(M-PPI) and lactose-coated dendrimer (L-PPI). All the synthesized dendrimeric systems were characterized by IR and NMR spectroscopy.

Haemolytic toxicity

A 2% red blood corpuscle (RBC) suspension was prepared in phosphate buffer saline (pH 7.4). PPI-5.0G and functionalized dendrimers were added to the RBC suspension (0.001 to 1 mg mL^{-1}) with Triton-X-100 as positive control. All the samples were incubated at 37°C for 1 and 4 h, and centrifuged at 3000 rpm for 15 min. The supernatant was analysed at 550 nm spectrophotometrically (1601 UV-Vis spectrophotometer, Shimadzu, Japan). The percentage of haemolysis was calculated by considering the absorbance of Triton-X-100 sample as 100% haemolysis. The experiment was carried out in triplicate.

Cytotoxicity

HepG2 and COS-7 cell lines were used for the assessment of the cytotoxicity of PPI-5.0G and its functionalized forms. Cells were seeded in 96-well microtitre plates at a density of 1×10^5 cells per millilitre in serum-containing media and left for 24 h for recovery. Test polymeric systems were added (0.001–1 mg mL^{-1}) in fresh complete media to microtitre plates and incubated for 24 or 72 h. Five hours before completion of the incubation period, 20 μL MTT (5 mg mL^{-1}) was added and the incubation was continued. The medium was removed and 100 μL DMSO was added to dissolve the formazan crystals. The optical density was measured at 550 nm using a plate reader (PowerWave X; BIO-TEK Instruments, Inc). The cell viability in the presence of polymers was expressed as the percentage of viability of cells in the absence of polymers. The experiment was carried out in triplicate.

The 50% cytotoxic concentration (CC$_{50}$) of polymeric systems against HepG2 and COS-7 at 72 h was determined.

Immunogenicity

The immunogenicity of synthesized polymers was evaluated in Balb/C mice (aged 1–2 months) (Kofta et al 2000). Animals were maintained in a 12-h light/dark cycle in a humidity and temperature-controlled animal house with free access to food and water. The animals were divided into seven groups of three animals each. On day 0, the test polymers (PPI-5.0G, DBG, DBPA, M-PPI and L-PPI) were separately administered to five groups intramuscularly as aqueous/poly(ethylene glycol 400) solutions (equivalent to1–4 mg of each polymer). The sixth group was kept as control. The seventh group was administered an equivalent dose of bovine serum albumin (BSA). Blood samples were collected 21 days post administration and antibody titre was monitored by ELISA. Ninety-six-well flat-bottomed microtitre plates were separately coated overnight at 4°C with 25 μg of test polymers. Non-specific binding was blocked by applying 100 μL well^{-1} of Tris buffer and 4% skimmed milk for 2 h at room temperature. The coating efficiency of the dendrimer in microtitre plates

was confirmed by washing the plates after 12 h with Tris buffer. The elute was scanned spectrophotometrically. The absence of any peak corresponding to PPI-5.0G or the functionalized polymers confirms the efficiency of the coating. Plates were washed with Tris buffer containing 0.25% Tween-40 (TBS-T). One hundred microlitres of sera diluted 100 times with 2% skimmed milk was added to each well. The plates were incubated at 37°C for 30 min and washed three times with TBS-T. One hundred microlitres of horse-radish peroxidase labelled goat-anti-mouse IgG monospecific antisera diluted 100 times in PBS-T was added to each well. The plates were incubated at 37°C for 30 min and washed three times with phosphate-buffered saline (pH 7.4) containing 5% Tween-20 (PBS-T) followed by addition of TMB as a substrate. The optical density was measured at 450 nm after 30 min using a plate reader (Labsystems, Finland).

The method was validated using calibration standards 0.00, 4.70, 6.5, 8.30, 11.4, 14.5, 27.0, 39.4, 58.3 and 77.2 ng mL^{-1}. In these, the intra-batch and inter-batch imprecision and inaccuracy of the method were below 19%. The lower limit for quantification was 6.00 ng mL^{-1} and the upper limit of quantification was determined to be 52.1 ng mL^{-1}. This is based on the application of highly specific monoclonal antibody against BSA. The BSA stock (1 mg mL^{-1}) solution was prepared in serum and appropriate dilutions were made from the same. The dilutions of BSA in serum were pipetted into the appropriate wells coated with anti-BSA monoclonal antibody (anti-BSA Mab, Axxora Life Sciences Inc. San Diego, CA). This antibody is specific to BSA and does not react with rat/rabbit albumin. Non-specific binding was blocked by applying 100 μL well^{-1} of Tris buffer and 4% skimmed milk for 2 h at room temperature. Plates were washed with Tris buffer containing 0.25% TBS-T. One hundred microlitres of sera containing different concentrations of BSA, diluted 100 times with 2% skimmed milk, was added to each well. The plates were incubated at 37°C for 30 min and washed three times with TBS-T. One hundred microlitres of horseradish peroxidase labelled goat-anti-rat IgG monospecific antisera diluted 100 times in PBS-T was added to each well. The plates were incubated at 37°C for 30 min and washed three times with PBS-T followed by addition of TMB as a substrate. The optical density was measured at 450 nm after 30 min using a plate reader.

Haematological studies

Healthy male albino rats (Sprague–Dawley strain) of uniform body weight (100 $\pm$ 20 g) with no prior drug treatment were used for all the present drug studies. The rats were maintained on standard diet and water. Five groups with three rats in each group were used to study the effect of test polymeric systems on haematological parameters. PPI-5.0G, DBG, DBPA, M-PPI and L-PPI were administered intravenously to five groups of rats separately as aqueous/poly(ethylene glycol 400) solutions (equivalent to 1–4 mg of each polymer). The sixth group, kept as control, was maintained on same regular diet for 7 days. Hematological parameters, i.e. white blood corpuscles (WBCs), RBCs, haemoglobin (Hb), haematocrit (HCT) and mean corpuscular haemoglobin (MCH), were determined in an Erma Particle Counter (Erma Inc, Tokyo, Japan).

Statistical analysis

One-way ANOVA follow by the Tukey–Kramer test was used for comparison of multiple sets of data. $P < 0.05$ was considered a statistically significant difference.

Results and Discussion

In the present study we carried out investigations of toxicological properties of some functionalized PPI dendrimers, which are expected to be promising drug carriers, and compared them with parent PPI dendrimer. The four functionalized PPI dendrimers used in this study are described in Table 1.

Synthesis and characterization of dendrimeric systems

The parent PPI dendrimer and its functionalized forms were synthesized, purified by dialysis and characterized by IR and NMR spectroscopy. The following spectroscopic data confirmed the synthesis of the different dendrimeric systems.

IR spectroscopy (Perkin-Elmer, Neat)
PPI-5.0G: $3369 \, cm^{-1}$ (–NH stretch, asymmetric), $3290 \, cm^{-1}$ (–NH stretch, symmetric), $1580 \, cm^{-1}$ (–NH bend)
DBG: $1628.9 \, cm^{-1}$ (amide I, CO stretch), $1570.1 \, cm^{-1}$ (amide II, –NH bend), $3336.9 \, cm^{-1}$ (–NH stretch), $2964 \, cm^{-1}$
DBPA: $1621.22 \, cm^{-1}$ (amide I, CO stretch), $1548.5 \, cm^{-1}$ (amide II, –NH bend), $3329 \, cm^{-1}$ (–NH stretch), $2928 \, cm^{-1}$ (–CH stretch)
M-PPI: $3352–3468 \, cm^{-1}$ (a broad band corresponding to –NH stretch and –OH stretch overlap), $2930 \, cm^{-1}$ (–CH stretch), $1629.65 \, cm^{-1}$ (amide I, CO stretch), $1494 \, cm^{-1}$ (amide II, –NH bend), $1146 \, cm^{-1}$ (C–H bend), $2561 \, cm^{-1}$ (SH stretch)
L-PPI: $3402–3548 \, cm^{-1}$ (a broad band corresponding to –NH stretch and –OH stretch overlap), $2919 \, cm^{-1}$ (–CH stretch), $1637.71 \, cm^{-1}$ (amide I, CO stretch), $1511 \, cm^{-1}$ (amide II, –NH bend), $1107 \, cm^{-1}$ (C–H bend), $2568 \, cm^{-1}$ (SH stretch)

NMR spectroscopy (DRX 300, 300 MHz, $CDCl_3$)
PPI-5.0G (D_2O): $\delta 2.928–2.490$ ppm (m, $–CH_2CH_2NH_2$), $\delta 1.942–1.718$ ppm (broad singlet, $–CH_2NH_2$)
DBG: $\delta 7.08$ ppm (broad singlet, –CONH), $\delta 1.14$ ppm (singlet, $[–CH_3]_3$ of t-BOC), $\delta 2.37$ ppm (triplet $–CH_2CONH–$), $\delta 1.286–1.452$ ppm (pentate, $–CH_2CH_2CH_2$), $\delta 1.579–1.722$ ppm (m, $–CH_2CH_2NHCO$), $\delta 3.07–3.14$ ppm (m, $–CH_2N(CH_2)_2$)
DBPA: $\delta 7.211$ ppm (broad singlet, –CONH), $\delta 3.645$ (singlet, *t*-butyl group), $\delta 3.212 \quad –3.084$ ppm (multiplet –

CH_2CH_2NHCO), $\delta 1.402–1.257$ ppm (pentate, $–CH_2CH_2CH_2$), $\delta 2.687–2.57$ ppm (broad doublet, $–CHCH_2C_6H_5$)
M-PPI: $\delta 2.46$, (m, $–CH_2CH_2NHCO$), $\delta 1.34–1.39$ ($–CH_2CH_2CH_2$), $\delta 2.49–2.86$ (m, $–SCH_2$), $\delta 7.83$ (broad singlet, $–CONH_2$), $\delta 3.40–\delta 4.1$ (broad singlet, hydroxyl groups of carbohydrate)
L-PPI: $\delta 2.95$, (m, $–CH_2CH_2NHCO$), $\delta 1.56–1.61$ ($–CH_2CH_2CH_2$), $\delta 2.80–3.02$ (m, $–SCH_2$), $\delta 7.87$ (broad singlet, $–CONH_2$), $\delta 3.47–\delta 4.03$ (broad singlet, hydroxyl groups of carbohydrate)

Haemolytic toxicity

The haemolytic toxicity of functionalized dendrimers was studied and compared to that of parent dendrimer. Unmodified PPI-5.0G displayed a concentration- and time-dependent haemolytic toxicity. This finding was in agreement with the results of Malik et al (2000), who reported haemolytic toxicity for diaminobutane- and diaminoethane-cored PPI dendrimers at concentrations above $1 \, mg \, mL^{-1}$. The haemolysis was clearly concentration and time dependent (Table 2). This haemolytic character could obviously be attributed to the polycationic nature of PPI-5.0G. Many reports indicating the haemolytic toxicity of polycations, including dendrimers, are available (Fischer et al 2003; Chen et al 2004). All the functionalized dendrimers investigated in the present study had terminal primary amines of the parent dendrimers masked with various chemical groups. Functionalization significantly reduced the hemolytic toxicity of the parent dendrimer at both time points ($P < 0.001$). Unlike the parent dendrimer, no concentration-dependent toxicity was observed in the case of functionalized dendrimers. The haemolytic toxicity profile of both modified amino-acid-coated dendrimers and carbohydrate-coated dendrimers was comparable ($P > 0.05$). This reduced haemolytic toxicity could be attributed to the absence of primary amines at the periphery. It was observed that anionic dendrimers have fewer haemolytic properties compared to cationic ones (Malik et al 2000). The literature suggests that protection of peripheral amine groups can greatly reduce the haemolytic profile of dendrimers. PEGylated, ester- or hydroxyl-terminated dendrimers with lesser haemolytic tendencies have also been synthesized (Chen et al 2004).

Cytotoxicity

The cytotoxicity of different dendrimeric systems was evaluated in COS-7 and HepG2 cell lines. The effect of terminal functional groups, concentration and incubation time on cytotoxicity was observed (Tables 3 and 4).

Table 1 Characteristics of polymeric systems used in the study

Serial no.	Polymeric system	Protecting moiety used	Peripheral groups	M. wt (kDa) (approx.)
1	PPI-5.0G	–	Primary amines	7
2	DBG	t-BOC-protected glycine	t-butyl	17
3	DBPA	t-BOC-protected phenylalanine	t-butyl	22
4	M-PPI	Mannose	Carbohydrate–OH	33
5	L-PPI	Lactose	Carbohydrate–OH	52

Table 2 Haemolytic toxicity after 1, 2 and 4 h of incubation

Conc. (mg mL^{-1})	Haemolysis (%)																	
	PPI-5.0G			DBG			DBPA			M-PPI			L-PPI			Dextran		
	1h	2h	4h	1h	2h	4h	1h	2h	4h	1h	2h	4h	1h	2h	4h	1h	2h	4h
0.001	11.4±0.3	26.8±0.2	59.6±0.4	3.6±0.6	3.6±0.3	3.6±0.5	3.1±0.5	3.5±0.3	3.9±0.2	2.8±0.2	3.0±0.1	3.1±0.3	2.7±0.2	2.6±0.3	2.6±0.1	2.4±0.4	2.5±0.3	2.7±0.4
0.01	17.1±0.4	36.2±0.4	71.1±0.3	3.4±0.2	3.8±0.3	4.1±0.4	3.1±0.4	3.1±0.2	3.2±0.3	2.9±0.3	2.9±0.2	2.7±0.1	2.9±0.2	2.9±0.2	2.9±0.5	2.2±0.5	2.3±0.5	2.6±0.6
0.1	26.7±0.3	48.9±0.3	74.8±0.5	2.9±0.3	3.6±0.5	4.1±0.5	2.6±0.4	3.1±0.5	4.2±0.5	2.6±0.1	2.6±0.3	2.6±0.2	2.4±0.4	2.4±0.1	2.5±0.3	2.3±0.2	2.3±0.2	2.3±0.1
1	34.2±0.2	51.6±0.3	86.2±0.6	3.2±0.2	3.8±0.5	4.9±0.2	3.2±0.2	3.2±0.3	3.3±0.4	3.0±0.2	2.9±0.5	2.9±0.6	2.6±0.1	2.5±0.2	2.2±0.3	2.4±0.3	2.4±0.3	2.5±0.4

All values are expressed as mean ± s.d. (n = 3).

Table 3 Cytotoxicity of polymeric systems against HepG2 at 24, 48 and 72 h

Conc. (mg mL^{-1})	Cell viability (%)														
	PPI-5.0G			DBG			DBPA			M-PPI			L-PPI		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
0.001	66.8±1.3	30.1±2.5	16.8±1.1	94.1±3.6	96.2±2.3	99.1±1.1	95.4±1.5	96.8±1.2	99.2±1.1	94.0±1.1	96.5±2.1	102.1±2.1	96.0±2.8	98.1±2.3	99.2±1.3
0.01	37.4±2.1	14.8±2.8	9.8±1.3	98.7±2.5	95.6±1.1	92.4±1.4	98±1.6	95.8±2.3	91.4±1.2	97.8±1.5	94.2±2.3	91.8±1.3	96.2±2.1	94.8±1.5	92.6±1.2
0.1	13.2±1.6	9.5±2.4	5.4±1.1	94.7±2.1	96.2±2.1	98.3±1.3	97.6±1.2	97.9±2.5	98.7±1.6	93.4±1.2	96.8±2.1	98.6±1.1	97.0±1.6	98.1±1.5	99.3±1.2
1	7.6±1.4	3.2±1.0	1.7±1.1	95.37±1.2	94.2±1.9	93.2±2.1	96.2±2.1	95.3±1.8	94.6±1.1	95.2±1.1	96.8±1.8	97.6±1.2	94.7±1.1	94.6±1.3	94.7±1.1

All values are expressed as mean ± s.d. (n = 3).

Table 4 Cytotoxicity of polymeric systems against COS-7 at 24, 48 and 72 h

Conc. (mg mL^{-1})	Cell viability (%)														
	PPI-5.0G			DBG			DBPA			M-PPI			L-PPI		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
0.001	71.5±1.2	42.6±2.6	23.1±2.1	98.0±2.6	95.6±2.3	93.6±1.2	95.0±1.5	93.5±0.8	92.8±1.1	96.4±1.2	94.5±1.1	93.3±1.7	94.0±1.6	95.2±2.4	96.5±1.2
0.01	47.3±2.3	25.9±2.1	14.6±1.5	98.8±2.1	94.8±2.5	92.6±1.3	99.3±2.1	95.8±1.5	91.7±2.1	103.0±3.6	95.8±1.5	92.4±1.1	99.6±1.3	92.5±1.5	89.9±2.3
0.1	18.3±2.1	14.7±2.9	9.8±1.1	96.2±1.6	93.6±1.6	91.8±2.1	95.9±1.2	95.2±1.1	94.4±1.6	98.5±1.5	95.4±1.2	91.9±1.4	96.1±1.6	94.3±1.4	92.5±1.5
1	11.7±1.5	5.9±1.5	2.3±1.2	96.1±1.2	93.6±1.4	92.0±1.3	93.0±1.0	92.5±1.6	92.1±1.1	98.1±1.2	97.4±1.3	96.7±1.2	97.7±1.8	96.2±1.1	95.8±1.3

All values are expressed as mean ± s.d. (n = 3).

The cytotoxicity was concentration and time dependent for PPI-5.0G. Cell viability at $0.001\,mg\,mL^{-1}$ at 24 h was 71.5% (COS-7) and 66.8% (HepG2), which reduced to 11.7% (COS-7) and 7.6% (HepG2) at $1\,mg\,mL^{-1}$ ($P < 0.001$). A similar statistically significant concentration-dependent cytotoxicity was also observed at 72 h. At any equivalent concentration in general, cell viability was significantly reduced on incubation for a longer period (72 h). At $1\,mg\,mL^{-1}$, for example, after 24 h incubation, 7.6 and 11.7% of cells of HepG2 and COS-7, respectively, remained viable. At the same concentration after 72 h viability was reduced to 1.7% (HepG2) and 2.3% (COS-7) ($P < 0.01$ for HepG2 and $P < 0.001$ for COS-7). The cytotoxicity was attributed to the presence of free primary amine groups in PPI-5.0G and the positive charge associated with them. The functionalized dendrimers, being devoid of free amine groups at their periphery, were not likely to carry the positive charge. PPI-5.0G after functionalization demonstrated a significantly reduced toxicity. DBG and DBPA ($1\,mg\,mL^{-1}$) demonstrated cell viabilities of 96.1 and 93%, respectively, in COS-7 cells after 24 h, and 92.04 and 92.1%, respectively, after 72 h. This was significantly higher than for PPI-5.0G ($P < 0.001$). Similar results were observed in HepG2 cells, where the viabilities corresponding to DBG and DBPA were 95.37 and 96.2%, respectively, after 24 h, and 93.21 and 94.66%, respectively, after 72 h ($P < 0.001$). M-PPI and L-PPI also demonstrated a superior toxicity profile to PPI-5.0G. Cell viabilities in COS-7 cells after 24 h were 98.1 and 97.71%, respectively, for M-PPI and L-PPI ($1\,mg\,mL^{-1}$) whereas after 72 h they were 96.72 and 95.83%. After 24 h the cell viabilities in HepG2 cells corresponding to M-PPI and L-PPI were 95.2 and 94.7%, respectively, and after 72 h they were 97.6 and 94.7%, respectively. In both the cell lines L-PPI and M-PPI demonstrated a superior toxicity profile compared to PPI-5.0G at both time points ($P < 0.001$). Functionalized dendrimers did not demonstrate any concentration- or time-dependent increase in cytotoxicity. At any equivalent concentration the cell viabilities at 24 and 72 h were not significantly different

($P > 0.05$). The cell viabilities at the lowest ($0.001\,mg\,mL^{-1}$) and highest ($1\,mg\,mL^{-1}$) concentrations were not significantly different at both the time points ($P > 0.05$).

The CC_{50} of polymeric systems against HepG2 and COS-7 at 72 h is shown in Table 5. The results once again confirm that functionalized dendrimers possess negligible cytotoxicity compared to the parent polymer (PPI-5.0G).

The influence of surface charge on cytotoxic properties is well documented. Malik et al (2000) reported higher cytotoxicity for cationic as compared to anionic dendrimers. Roberts et al (1996) also observed charge-dependent toxicity of dendrimers during their investigation of PAMAM dendrimers. Protection of surface amine groups reduces the inherent cytotoxicity of the cationic dendrimers. Dendrimer–DNA or dendrimer–oligonucleotide complexes were found to be less cytotoxic than the parent dendrimers (Brazeau et al 1998; Yoo & Juliano 2000). Jevprasesphant et al (2003) observed reduced cytotoxicity of PAMAM (G2, G3, G4) dendrimers on PEGylation with PEG 2000. Attachment of lauryl chains to the dendrimer periphery also caused a significant reduction in cytotoxicity. Other reports describing a reduction in the cytotoxicity of polycationic dendrimers on PEGylation are available (Luo et al 2002). Our observations are in good agreement with the available literature.

Immunogenicity

The ELISA technique used in the present study was validated using calibration standards 0.00, 4.70, 6.5, 8.30, 11.4, 14.5, 27.0, 39.4, 58.3 and $77.2\,ng\,mL^{-1}$. Under these conditions the intra-batch and inter-batch imprecision and inaccuracy of the method were below 19%. The lower limit of quantification was $6.00\,ng\,mL^{-1}$ and the upper limit of quantification was determined to be $52.1\,ng\,mL^{-1}$. The validation data clearly indicate that the method employed is valid and consistency between the batches is very high (Table 6). The immunogenicity of dendrimeric systems was investigated in Balb/C mice. None of the polymers elicited detectable antibody titre (IgG) at 450 nm at

Table 5 CC_{50} of polymeric systems against HepG2 and COS-7 at 72 h

Cell line	CC_{50} (mg mL^{-1})				
	PPI-5.0G	DBG	DBPA	M-PPI	L-PPI
HepG2	0.00058 ± 0.0002	0.0553 ± 0.0001	0.0615 ± 0.0002	0.0235 ± 0.0004	0.0619 ± 0.0004
COS-7	0.00062 ± 0.0001	0.0574 ± 0.002	0.0721 ± 0.003	0.0246 ± 0.0005	0.0589 ± 0.0003

All values are expressed as mean $\pm$ s.d. (n = 3).

Table 6 Validation of ELISA technique employed for evaluation of immunogenicity of polymeric systems

Variation	Parameter (%)	BSA concentration (ng mL^{-1})			
		6.00	29.6	41.5	52.1
Intra-batch	Imprecision	5.18	2.33	4.76	2.12
Intra-batch	Inaccuracy	4.33	−1.01	−18.55	−14.18
Inter-batch	Imprecision	17.94	2.17	6.18	3.10
Inter-batch	Inaccuracy	0.33	4.05	−13.01	−15.74

Table 7 Influence of polymeric systems on haematological parameters

Polymeric systems	Hematological parameters				
	RBC	WBC	Hb	HCT	MCH
Control	7.15 ± 1.37	8.25 ± 1.67	14.89 ± 1.35	41.69 ± 5.51	20.76 ± 1.35
PPI-5.0G	4.43 ± 1.15	12.01 ± 1.23	8.26 ± 1.39	27.06 ± 3.16	18.82 ± 1.06
DBG	7.01 ± 1.41	9.01 ± 1.02	12.76 ± 1.31	38.71 ± 3.18	18.15 ± 0.78
DBPA	6.99 ± 1.22	8.99 ± 1.36	12.51 ± 1.35	38.38 ± 3.14	18.01 ± 1.08
M-PPI	7.36 ± 1.01	8.24 ± 1.89	13.46 ± 1.78	41.82 ± 4.32	18.41 ± 0.53
L-PPI	7.24 ± 1.24	8.37 ± 1.46	13.01 ± 1.59	41.02 ± 4.62	18.15 ± 1.21

All values are expressed as mean ± s.d. (n = 3).

any concentration tested. This clearly shows that the dendrimers under study were unable to elicit any detectable humoral immune response under the experimental conditions. This also suggests that the polymers were treated as 'native' by the host immune system. However, the polymers were not tested for cell-mediated immune response because of their size and non-particulate nature. The long-term effect on the immune system of repeated administration is yet to be tested. Roberts et al (1996) investigated the immunogenicity of PAMAM dendrimers (generation 3, 5 and 7) by immunoprecipitation and Ouchterlony double-diffusion assay and observed no signs of immunogenicity at a dose range of 1×10^{-1} to $1 \times 10^{-4} \mu$M. Our observation was in accordance with this report.

Haematological study

The influence of the parent PPI-50G and the functionalized dendrimeric systems on different haematological parameters was evaluated (Table 7). A statistically significant difference was observed between the RBC and WBC counts of the PPI-5.0G treated group and control. This observation could be due to the polycationic nature of the uncoated dendrimer. The RBC count was significantly lower, while the WBC count was significantly higher ($P < 0.05$). This observation is in agreement with the previously available report on galactose-coated dendrimer (Kofta et al 2000). A corresponding decline in Hb content and MCH value was also observed. There was a significant difference in HCT value between control and PPI-5.0G. Functionalized dendrimers did not show any statistically significant difference in the value of haematological parameters compared to control. This indicates that functionalization improves the biocompatibility of PPI-5.0G.

Conclusions

PPI-5.0G and its functionalized forms were tested for their toxicity profile. The results endorsed the positive-charge associated toxicity of dendrimers. Masking peripheral amine with various protecting groups could reduce the cytotoxicity, as observed in this study. All the polymers tested were found to be non-immunogenic. This improved biocompatibility could be of particular interest as the proposed functionalized dendrimers have potential application as drug carriers. Thus amino-acid-protected and carbohydrate-coated PPI dendrimers could make newer biocompatible options available for controlled and targeted drug delivery.

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